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(54) Chemiluminescent method and compositions.

(57) A chemiluminescent method using a protected enhancer compound which is triggered by a hydrolytic enzyme and then enhances a chemiluminescent reaction is described. The reaction involves an amino substituted acylhydrazide which is activated to produce light by a peroxide compound and a peroxidase in the presence of the activated enhancer compound. The result is enhanced generation of light from the reaction. The hydrolytic enzyme is present alone or as a label in an immunoassay, DNA probe assay or other assay wherein the hydrolytic enzyme is bound to a reporter molecule.

EP 0 516 948 A1

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a method of generating light chemically (chemiluminescence) by the action of an enzyme which generates an enhancer for a second enzyme from a protected enhancer where the second enzyme, in turn, catalyzes a chemiluminescent reaction. The invention also relates to the use of this method to detect the first enzyme. Further, the invention relates to the use of the method to detect and quantitate various biological molecules bound to the first enzyme. For example, the method may be used to detect the first enzyme singly or as a label for haptens, antigens and antibodies by the technique of immunoassay, proteins by Western blotting, DNA and RNA by Southern and Northern blotting, respectively. The method may also be used to detect DNA in DNA sequencing applications.

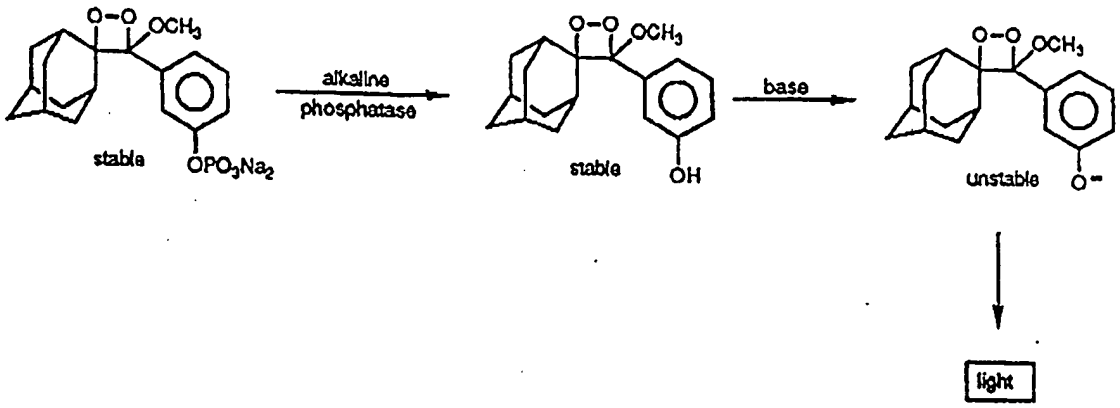
2. Description of the Prior Art

a. Chemiluminescent oxidation of luminol and related compounds. The amino-substituted cyclic acylhydrazides such as luminol and isoluminol react with H_2O_2 and a peroxidase enzyme catalyst (such as horseradish peroxidase, HRP) under basic conditions with emission of light. This reaction has been used as the basis for analytical methods for the detection of H_2O_2 and for metal ions. Luminol and isoluminol may be directly conjugated to a species to be detected. The first chemiluminescent immunoassay using luminol as a label was reported by Schroeder for an assay of biotin (H. R. Schroeder, P. O. Vogelhut, R. J. Carrico, R. C. Boguslaski, R. T. Buckler, Anal. Chem., 48, 1933 (1976)). Several applications of the use of luminol derivatives as labels have been reported since then (H. R. Schroeder in Luminescent Immunoassays: Perspectives in Endocrinology and Clinical Chemistry, M. Serio and M. Pazzagli, Eds., Raven Press, New York, pp 129-146 (1982); M. Pazzagli, G. Messeri, A. L. Caldini, G. Monetti, G. Martinazzo, M. Serio, J. Steroid Biochem., 19, 407 (1983); Bioluminescence and Chemiluminescence New Perspectives, J. Scholmerich, et al, Eds., J. Wiley & Sons, Chichester (1987)). Various enhancers have also been employed in conjunction with the use of luminol to increase the intensity of light emitted. These include D-luciferin (T. P. Whitehead, G. H. Thorpe, T. J. Carter, C. Groucutt, L. J. Kricka, Nature, 305, 158 (1983)) and p-iodophenol and p-phenylphenol (G. H. Thorpe, L. J. Kricka, S. B. Mosely, T. P. Whitehead Clin. Chem., 31, 1335 (1985)).

b. Enzyme-Catalyzed Chemiluminescent Reactions

1. Enzymatic Triggering of Stabilized 1,2-Dioxetanes. Recently developed thermally stable dioxetanes can be triggered by chemical and enzymatic processes to generate chemiluminescence on demand (A. P. Schaap, U.S. Patent No. 4,857,652; A. P. Schaap, R. S. Handley, B. P. Giri, Tetrahedron Lett., 935 (1987); A. P. Schaap, T. S. Chen, R. S. Handley, R. DeSilva, B. P. Giri, Tetrahedron Lett., 1155 (1987); and A. P. Schaap, M. D. Sandison, R. S. Handley, Tetrahedron Lett., 1159 (1987)). These dioxetanes exhibit several key features: (1) the stabilizing influence of spiro-fused adamantyl groups has been utilized to provide dioxetanes that have "shelf lives" of years at ambient temperature and (2) an electron-rich aromatic group which allows the efficient production of excited states and which produces fluorescence when excited and (3) a protecting group which prevents the chemiluminescent decomposition until removed by an enzyme or other chemical reaction.

Scheme 1



For example, enzymatic triggering by alkaline phosphatase was observed with the phosphate-substituted dioxetane (1) derived from methyl 3-hydroxybenzoate and adamantanone (Lumigen® PPD). Addition of the enzyme to a solution of dioxetane results in chemiluminescence emitted over several minutes. The total light emission was found to be linearly dependent on the dioxetane concentration. The rate of decay of the emission is a function of enzyme concentration while the total light emission is independent of the enzyme concentration. As a result of the very low background luminescence from slow hydrolysis of the dioxetane in the buffer, in the presence of enhancers less than 10^{-18} mol (1 attomol) of alkaline phosphatase can be detected (A. P. Schaap, H. Akhavan, L. J. Romano, Clin. Chem., 35, 1863 (1989)). Similar protected dioxetane substrates for other enzymes, namely beta-galactosidase, esterase and sulfatase have also been developed. A number of commercial products for detection of proteins and nucleic acids are sold employing chemiluminescent detection of alkaline phosphatase with Lumigen® PPD (D. Pollard-Knight, A. C. Simmonds, A. P. Schaap, H. Akhavan, M. A. W. Brady, Anal. Biochem., 185, 353-358 (1990); J. M. Clyne, J. A. Running, R. Sanchez-Pescador, D. Besemer, M. Stempien, A. P. Schaap, R. S. Stephens, M. S. Urdea, J. Biolumin. Chemilumin. 2, 193 (1988)).

2. Enzymatic generation of hydrogen peroxide. Various enzymatic reaction schemes are known which produce hydrogen peroxide. The generated hydrogen peroxide can, in turn, be used to oxidize a compound which emits light. For example, glucose oxidase reacts with O_2 and glucose to produce H_2O_2. Similarly, amino acid oxidase reacts with an amino acid and O_2 to produce H_2O_2. Examples of compounds which are oxidized by hydrogen peroxide to produce light are luminol and isoluminol, lucigenin, esters of N-methylacridine and esters and amides of oxalic acid. Glucose-6-phosphate dehydrogenase and galactose-6-phosphate dehydrogenase have been used to produce H_2O_2 indirectly by reduction of oxygen through an electron-relay system (K. Tanabe, T. Kawasaki, M. Maeda, A. Tsuji, M. Yabuuchi, Bunseki Kagaku, 36, 82 (1987); A. Tsuji, M. Maeda, H. Arakawa, Anal. Sci., 5, 497 (1989)).

3. Enzymatic generation of luminol from a luminol-NAG conjugate. The compound o-aminophthalydrazide-N-acetyl-beta-D-glucosaminide (luminol-NAG) is a substrate for the enzyme N-acetyl-beta-D-glucosaminidase which serves as a masked form of luminol. Upon action of the enzyme on this substrate, luminol is liberated which may be detected as described above (K. Sasamoto, Y. Ohkura, Chem. Pharm. Bull., 38(5), 1323 (1990)).

4. Bioluminescent detection of firefly or bacterial luciferase. A class of enzymes known as luciferases, catalyze the autooxidation of certain substrates known as luciferins in a number of living organisms. An example is the firefly whose bioluminescent process oxidizes its luciferin to produce light with 88% efficiency. A DNA dot-hybridization assay has been reported in which alkaline phosphatase was employed to generate free firefly luciferin from the phosphate salt. Luciferase added in the final step caused the chemiluminescent oxidation of the luciferin (R. Huber, R. Geiger, Nuc. Ac. Res., 16(3), 1213 (1988)). Only one enzyme amplification step is involved in this method since the first enzyme only generates a substrate for the second enzyme.

c. Use of Chemiluminescence in Enzyme Immunoassays and DNA Hybridization Assays. Biological assays such as enzyme immunoassays and DNA probe assays involving enzymes utilize a wide variety of substrates which either form a color (chromogenic), become fluorescent (fluorogenic) or emit light (chemiluminogenic) upon reaction with the enzyme. Of these three choices, chemiluminescence offers the greatest sensitivity. In an assay, the enzyme (reporter enzyme) is conjugated or bound to the molecule to be detected or to some other substance capable of selectively binding or associating with the molecule to be detected. Once the bound reporter enzyme is separated from unbound enzyme, a substrate is provided with which the reporter enzyme generates a signal. Chemiluminogenic substrates used to date include enzyme-triggerable dioxetanes such as the alkaline phosphatase substrate Lumigen® PPD. This substrate has been used extensively in enzyme-linked immunoassays and DNA probes. The enzyme horseradish peroxidase has been widely used in enzyme immunoassays and DNA hybridization assays with chemiluminescent detection using luminol or isoluminol as substrate (T. P. Whitehead, G. H. Thorpe, T. J. Carter, C. Groucutt, L. J. Kricka, Nature 305, 158 (1983); G. H. Thorpe, L. J. Kricka, S. B. Mosely, T. P. Whitehead, Clin. Chem., 31 1335 (1985); G. H. Thorpe, S. B. Mosely, L. J. Kricka, R. A. Stott, T. P. Whitehead, Anal. Chim. Acta, 170, 107 (1985); J. A. Matthews, A. Batki, C. Hynds, L. J. Kricka, Anal. Biochem., 151, 205, (1985)). Commercially available kits for conjugation of HRP with enhanced luminol chemiluminescent detection are sold under the tradename Amerlite™.

d. Enzyme cascade sequences for sensitive detection. A coupled enzyme cascade reaction for the colorimetric detection of alkaline phosphatase has been reported (D. M. Obzansky, D. M. Severino, Abstracts of 23rd Oak Ridge Conference on Advanced Analytical Concepts for the Clinical Laboratory, April 12, 1991). In this scheme, alkaline phosphatase generates a substance which reacts with an inactive form of a second enzyme, converting it to its active state. The second enzyme reacts with its own substrate producing H_2O_2. The H_2O_2 is detected through a subsequent colorimetric procedure.

A colorimetric enzyme immunoassay method has been reported wherein HRP bound in a sandwich immunoassay catalyzes the oxidative deposition of a biotin-phenol conjugate with H_2O_2. The oxidized product becomes deposited on the solid surface of the assay vessel. The bound biotin is used to capture additional enzyme-streptavidin conjugate. The captured enzyme may be either more HRP or a different enzyme, i.e. alkaline phosphatase or beta-galactosidase. No chemiluminescent detection was used (M. N. Bobrow, T. D. Harris, K. J. Shaughnessy, G. J. Litt, J. Immun. Meth., 125, 279 (1989)).

A method is known which features the enzymatic release of an enzyme inhibitor Anal. Biochem., 179, 229 (1989). The basis of this method is the enzymatic release of an inhibitor to a second enzyme. Sensitivity shows an inverse dependence on concentration of the first enzyme and is limited to the sensitivity with which the second enzyme can be detected. No true amplification is achieved by the use of two enzymes.

Glucose-6-phosphate dehydrogenase and galactose-6-phosphate dehydrogenase have been used to produce H_2O_2 indirectly by reduction of oxygen through an electron-relay system (K. Tanabe, T. Kawasaki, M. Maeda, A. Tsuji, M. Yabuuchi, Bunseki Kagaku, 36, 82 (1987); A. Tsuji, M. Maeda, H. Arakawa, Anal. Sci., 5, 497 (1989)). In these procedures, enhancement results only from the rapid recycling of the enzyme, not from the generation of another catalytically active species.

U.S. Patent No. 4,318,980 discloses a heterogeneous binding assay employing a catalytic cycling agent (nicotinamide adenine dinucleotide, NAD) as the label for the analyte. The cycling agent is subsequently detected by a chemiluminescent reaction. Only a single enzyme amplification step results.

U.S. Patent No. 4,598,042 discloses an immunoassay for phosphatase conjugates wherein the phosphatase converts NADP to NAD. The NAD formed while cycling between its oxidized and reduced forms acts as a cofactor for alcohol dehydrogenase cycling agent and catalyzes the formation of a colored compound. The use of chemiluminescence or peroxidases is not disclosed.

U.S. Patent 4,498,042 discloses an immunoassay which uses as a label an enzyme which converts an inactive form of a second enzyme to its active form. The second enzyme is detected by reaction with a substrate. Alternatively the second enzyme may react to generate a third enzyme which is subsequently detected by reaction with a substrate.

3. Summary

The detection of chemiluminescence from the oxidation of an amino-substituted cyclic acylhydrazide by a peroxide compound catalyzed by a peroxidase enzyme can be accomplished with good sensitivity. Enhancement of this reaction by incorporation of an enhancer has permitted the measurement of chemiluminescence using still lower levels of the peroxidase enzyme. Coupling this enzyme to a biological molecule of interest then permits the detection of this biological molecule with great sensitivity. This

technology limits the utility specifically to the use of only a peroxidase enzyme. A need exists to make use of this enhanced catalytic generation of amino-substituted cyclic acylhydrazide chemiluminescence by initiation with other enzymes. By making another enzyme the detection marker or label in this system, the result is a further amplification of the enzyme-amplified chemiluminescent signal. This amplification-of-amplification will lead to detection of increasingly lower amounts of enzyme and, hence, to the biological molecule of interest. A key consideration in developing ultrasensitive detection systems is to maintain the lowest possible level of background signal in relation to the signal to be measured. It is of paramount importance in any proposed detection method to provide the largest signal possible through amplification without a concomitant increase in background signal (i.e. only the signal of interest should be amplified).

OBJECTS

It is therefore an object of the present invention to provide a method and enzyme-triggerable protected enhancers for use in generating chemiluminescence by the action of a peroxidase enzyme for the detection of biological materials and compounds. Additionally, it is an object of the present invention to provide a method and enzyme-triggerable protected enhancers for use in generating chemiluminescence by the action of a peroxidase enzyme for use in immunoassays in solution. Further, it is an object of the present invention to provide a method and enzyme-triggerable protected enhancers for use in generating chemiluminescence by the action of a peroxidase enzyme for detection of proteins in Western blots and DNA in Southern blots and other DNA hybridization assays.

IN THE DRAWINGS

Figure 1 is a plot of chemiluminescence intensity as a function of time using the reaction system described in Example 1. Chemiluminescence was measured in a Turner Designs model TD20-e luminometer at ambient temperature. Light was attenuated with a 200x neutral density filter. The reaction was initiated by addition of alkaline phosphatase.

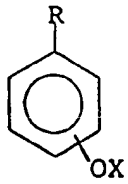
Figure 2 shows chemiluminescent Western blot of human transferrin. Experimental conditions for the Western blot are described in the text. Tracks were loaded with (left to right) 5 ng, 1 ng, 200 pg, 50 pg, and 20 pg human transferrin. Washed and blocked membrane was incubated in the reagent for 5 minutes and exposed to KODAK X-OMAT AR film for 5 seconds.

GENERAL DESCRIPTION

The present invention relates to a method for generating light which comprises mixing: a hydrolytic enzyme; and a protected enhancer compound, a peroxide compound and an amino substituted acylhydrazide which generates light upon reaction with the peroxide compound and the peroxidase enzyme, wherein the protected enhancer compound has the formula ArOX wherein X is a leaving group which is reactive with the hydrolytic enzyme and Ar is a non-interfering aromatic ring group which can contain C, O, S or N in the ring, wherein the hydrolytic enzyme reacts with the protected enhancer compound to remove X and thereby enhance the level of light produced by the amino substituted acylhydrazide as compared to the level of light without the enhancer compound.

The present invention particularly involves a method of generating a catalyst or cycling agent by the action of a first enzyme which enhances or amplifies the action of a second enzyme which, in turn, catalyzes a chemiluminescent reaction. The invention also relates to the use of this method to detect the first enzyme with high sensitivity. Further, the invention relates to the use of the method to detect and quantitate various biological molecules which are bound to this enzyme by chemical bonds or through physical interactions. The intensity of the resulting chemiluminescence provides a direct measure of the quantity of labeled organic or biological molecule. For example, the method may be used to detect haptens, antigens and antibodies by the technique of immunoassay, proteins by Western blotting, DNA and RNA by Southern and Northern blotting, respectively. The method may also be used to detect DNA in DNA sequencing applications.

Preferably the compound is a phenolic compound of the formula



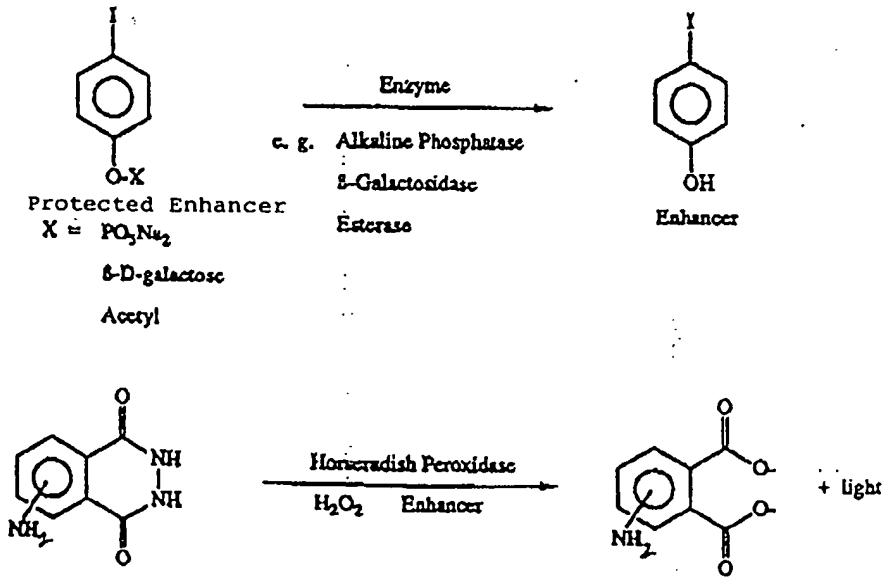
wherein R is a non-interfering group which may be heterocyclic and may also be joined to other parts of the benzene ring.

Thus R can be selected from the group consisting of a halogen (Cl, Br, Fl or I), alkyl containing 1 to 30 carbon atoms (which can be cyclic), aryalkyl containing 1 to 30 carbon atoms and wherein alkylaryl or aralkyl can be substituted with halogen, O, N or S in place of a carbon.

Further the present invention relates to a kit for detecting a hydrolytic enzyme in an assay which comprises a protected enhancer compound, a peroxide compound, a peroxidase and an amino substituted acylhydrazide which generates light upon reaction with the peroxide compound and the peroxidase enzyme, wherein the enhancer compound has the formula ArOX wherein X is a leaving group which is reactive with the hydrolytic enzyme and Ar is a non-interfering aromatic ring group which can contain O, S or N in the ring, wherein the hydrolytic enzyme reacts with the enhancer compound to remove X and thereby enhance the level of light produced by the amino substituted acylhydrazide as compared to the level of light without the enhancer compound.

Finally the present invention relates to a composition for reaction with a hydrolytic enzyme which comprises: an amino-substituted acylhydrazide, and a protected enhancer compound having the formula ArOX wherein X is a leaving group which is reactive with the hydrolytic enzyme and Ar is a non-interfering aromatic group which can contain O, S or N in the ring, wherein the hydrolytic enzyme reacts with the enhancer compound to remove X and thereby enhances the level of light produced by the amino substituted acylhydrazide in the presence of a peroxide compound and a peroxidase as compared to the level of light without the enhancer compound.

Scheme 2



Removal of an X group from a protected enhancer chemical compound by a hydrolytic enzyme

generates an enhancer compound, such as a phenolic compound, which greatly enhances the ability of a second enzyme, namely horseradish peroxidase to catalyze the oxidation of luminol with hydrogen peroxide to produce light. Several examples of masked or protected enhancer compounds (ArOX) are shown with specific leaving groups X. Various specific enzymes react with the protected enhancer compounds by cleaving the X group and liberating the arylhydroxy compound or aryloxide ion. Possible X groups include any chemical leaving group which is stable under the conditions of use and may be subsequently removed by treatment of the labelled molecule bearing the group X with the appropriate enzyme. The corresponding OX groups include but are not limited to alkyl or aryl carboxyl ester, inorganic oxyacid salt, and oxygen-pyranoside.

An integral component of the invention is that an amino-substituted cyclic acylhydrazide, a peroxide compound, a peroxidase enzyme and a suppressing agent may be combined in one solution and stored for later use without generating a large background chemiluminescent signal. This would not be expected in view of the fact that the combination of an amino-substituted cyclic acylhydrazide, hydrogen peroxide and a peroxidase enzyme normally constitute a highly chemiluminescent reaction system. Further, incorporation of the protected catalyst to this mixture may also be accomplished without generating a large background chemiluminescent signal.

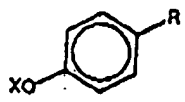
The invention relates to a method for generating chemiluminescence by the action of an enzyme or enzyme conjugate with a mixture containing an amino-substituted cyclic acylhydrazide, a peroxide compound, a peroxidase enzyme, a protected enhancer compound (ArOX) and a suppressing agent in an aqueous buffer. The invention also relates to the use of the method for the detection of biological molecules such as haptens, antigens, antibodies, and nucleic acids in solution. The invention also relates to the use of the method for the detection on solid supports such as nitrocellulose or nylon membranes, of proteins in Western blots, DNA in Southern blots and other DNA hybridization assays and RNA in Northern blots.

The present method relates to a protected enhancer compound represented by the formula Ar-OX selected from the compounds in Table 1 with X in place of H in OH and wherein Ar is a non-interfering group selected from an aryl, aralkyl, or heteroaryl group, and wherein X is a chemically labile substituent which is removed by an enzyme so that arylhydroxy compound or aryloxide ion is produced and wherein OX is selected from the group consisting of alkyl or aryl carboxyl ester, inorganic oxyacid salt, and oxygen-pyranoside.

Table 1

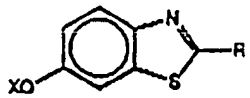
Aromatic Enhancer Compound (ArOH)	
Firefly luciferin Dehydroluciferin 6-Hydroxybenzothiazole 2-Cyano-6-hydroxybenzothiazole p-Iodophenol o-Iodophenol p-Bromophenol	o-Phenylphenol p-Hydroxycinnamic acid o-Hydroxycinnamic acid 2-Chloro-4-phenylphenol 2-Naphthol 1-Bromo-2-naphthol 1,6-Dibromo-2-naphthol
p-Chlorophenol 2,4-Dichlorophenol	p-Hydroxyphenylacetic acid
p-Phenylphenol	o-Hydroxyphenylacetic acid

The present method preferably relates to a protected enhancer compound represented by the formula

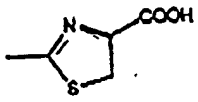


wherein R is selected from the group consisting of I, Ph, or -HC=CH-COOH, wherein X is a chemically labile substituent which is removed by an enzyme so that the phenol or phenolate ion is produced and wherein OX is selected from the group consisting of alkyl or aryl carboxyl ester, inorganic oxyacid salt, and oxygen-pyranoside.

The present method particularly relates to a protected enhancer compound represented by the formula



wherein R is selected from the group consisting of H, CN, or



wherein X is a chemically labile substituent which is removed by an enzyme so that the phenol or phenolate ion is produced and wherein OX is selected from the group consisting of alkyl or aryl carboxyl ester, inorganic oxyacid salt, and oxygen-pyranoside.

The present invention involves a solution in an aqueous buffer containing (1) a protected enhancer compound, (2) a peroxide compound wherein the peroxide compound may be hydrogen peroxide, a perborate salt, urea peroxide or an organic peracid, (3) a peroxidase enzyme, wherein the enzyme may be horseradish peroxidase, microperoxidase or lactoperoxidase, (4) an amino-substituted cyclic acylhydrazide, wherein the amino-substituted cyclic acylhydrazide may be selected from the group consisting of 3-aminophthalhydrazide (luminol), 3-(N-alkylamino)phthalhydrazide,3-(N,N-dialkylamino)phthalhydrazide, 6-alkyl-3-aminophthalhydrazide, 4-aminophthalhydrazide(isoluminol), 4-(N-alkylamino)phthalhydrazide, 4-(N,N-dialkylamino)phthalhydrazide, 7-aminophthalhydrazide, 7-(N-alkylamino)naphthalhydrazide 7-(N,N-dialkylamino)naphthalhydrazide and (5) a suppressing agent wherein the agent may be selected from the group consisting of non-fat milk, I-block solution, bovine serum albumin, gelatin or various surfactants including non-ionic, cationic, anionic and zwitterionic surfactants.

SPECIFIC DESCRIPTION

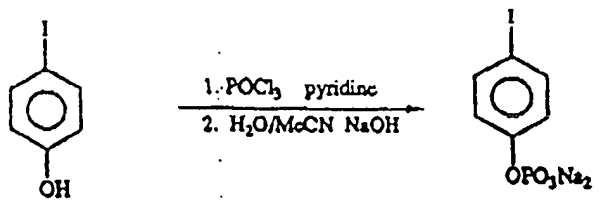
Instrumentation

Nuclear magnetic resonance (NMR) spectra were obtained on a General Electric QE300™ spectrometer as solutions in $CDCl_3$ with tetramethylsilane as internal standard unless noted otherwise. Mass spectra were obtained on either a Kratos MS-80™ or an AEI MS-90™ spectrometer. Weights were obtained on a Mettler AE 163™ analytical balance. Chemiluminescence measurements were performed using either a Turner TD 20e luminometer or a device built in this laboratory which is interfaced to an Apple Macintosh™ computer.

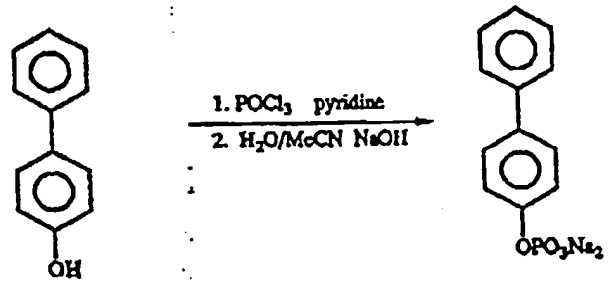
Materials

Solvents and reagents obtained from various commercial sources were the best available grade and were used without further purification unless noted otherwise. Solvents were dried by distillation from sodium or calcium hydride. Enzymes were purchased from commercial sources and used without further purification. Horseradish peroxidase IgG conjugate was purchased from Cappel Products. The conjugate was used for these studies rather than free commercial enzyme because free enzyme showed phosphatase activity.

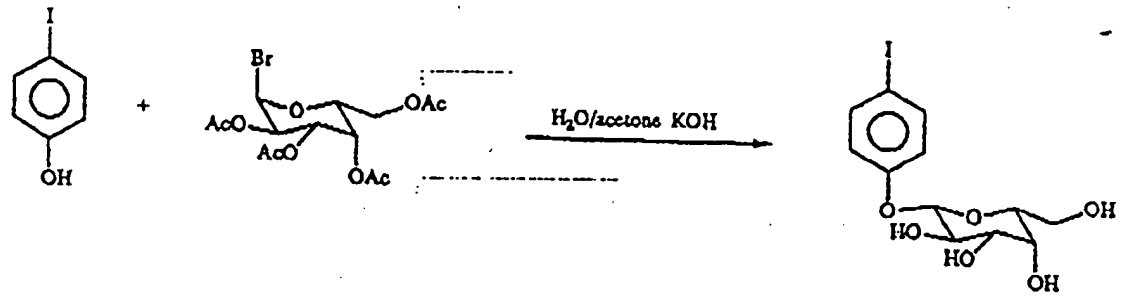
Synthesis of Enzyme Substrates



p-Iodophenylphosphate, disodium salt. Pyridine (0.395, g, 5 mmol) was dissolved in 15 mL of CH_2Cl_2. The solution was cooled to about 5 °C and stirred while $POCl_3$ (2.30 g, 15 mmol) was added slowly. Next, a solution of p-iodophenol (1.10 g, 5 mmol) in 10 mL of CH_2Cl_2 was added dropwise over a 5 minute period. The cooling bath was removed and stirring continued for 30 minutes. The solvents were removed under reduced pressure and 15 mL of CH_3CN was added to dissolve the solids. A solution of NaOH (0.80 g, 20 mmol) in 1 mL of water was added dropwise with stirring causing a white precipitate. After standing 10 minutes, the solids were collected and washed with a large volume of CH_3CN followed by acetone and dried in the air.

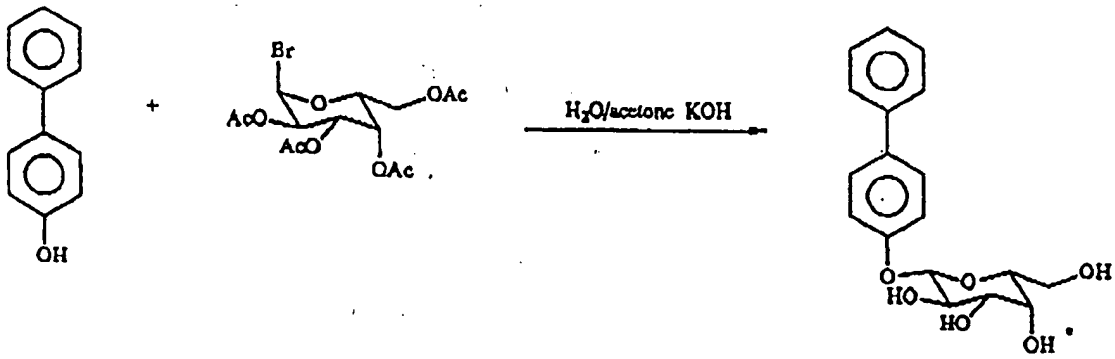


p-Phenylphenol phosphate, disodium salt. Pyridine (0.395 g, 5 mmol) was dissolved in 15 mL of CH_2Cl_2.The solution was cooled to about 5 °C and stirred while $POCl_3$ (2.30 g, 15 mmol) was added slowly. Next, a solution of p-phenylphenol (0.85 g, 5 mmol) in 10 mL of CH_2Cl_2 wasadded dropwise over a 5 minute period. The cooling bath was removed and stirring continued for 30 minutes. The solvents were removed under reduced pressure and 15 mL of CH_3CN was added to dissolve the solids. A solution of NaOH (0.80 g, 20 mmol) in 1.2 mL of water was added dropwise with stirring causing a white precipitate. After standing 10 minutes, the solids were collected and washed with a large volume of CH_3CN followed by acetone and dried in the air.

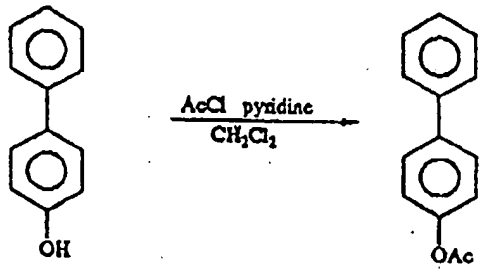


p-Iodophenyl-beta-D-galactopyranoside. p-Iodophenol (1g, 4.5 mmol) dissolved in 3 mL of acetone was treated with 3 mL of 5 M KOH (aq). Acetobromogalactose (5.6 g, 13.6 mmol) was added in portions to the stirred solution. Initially a 4.1 g portion was added with 1 ml of 5 M KOH. At two-three hour intervals 0.5 g portions of acetobromogalactose was added accompanied by 0.5 ml of 5 M KOH until a total of 5.6 g (3 eq.) were added. Stirring was continued overnight. Water was added and the solution extracted with methylene chloride followed by ethyl acetate. The combined organic layers were evaporated and the crude product

purified by column chromatography on silica using 30% ethyl acetate in methanol to remove unreacted sugar. Removal of solvents from the appropriate fractions produced the tetraacetate ester of the desired compound. Hydrolysis to the desired compound was achieved by dissolving a 300 mg portion in 2 mL of acetone and stirring with 650 µL of 10 M KOH overnight. The acetone was evaporated and 30 mL of water added. Ammonium chloride was added to neutralize the pH and the resulting solution extracted with ethyl acetate. Evaporation of the ethyl acetate after column chromatography on silica with 1:1 ethyl acetate/methanol.



p-Phenylphenol-beta-D-galactopyranoside. p-Phenylphenol (0.25 g, 1.5 mmol) dissolved in 1.5 mL of acetone was treated with 0.5 mL of 10 M KOH (aq). Acetobromogalactose (1.8 g, 4.4 mmol) was added in portions to the stirred solution. Initially a 1.5 g portion was added with 0.5 ml of 10 M KOH. After two hours a 0.3 g portion of acetobromogalactose was added accompanied by 0.5 ml of 10 M KOH. Stirring was continued overnight. TLC indicated that the starting material was consumed. Another 1 mL of 10 M KOH was added and stirring continued for 1 day. The acetone was evaporated and the residue extracted 5 times with ethyl acetate. The combined organic layers were dried, evaporated and the crude product purified by column chromatography on silica using 30% methanol in ethyl acetate.



p-Phenylphenol acetate. Pyridine (2 mL) was dissolved in 15 mL of CH_2Cl_2. The solution was cooled to about 5°C and stirred while acetyl chloride (1.8 g, 4.4 mmol) was added slowly. Next, a suspension of p-phenylphenol (0.85 g, 5 mmol) in 20 mL of CH_2Cl_2 was added dropwise over a 15 minute period. The cooling bath was removed and stirring continued for 30 minutes. The solvents were removed under reduced pressure and 50 mL of ethyl acetate was added to dissolve the solids. The solution was extracted with water, dried over Na_2SO_4 and evaporated under reduced pressure.

Chemiluminescent Detection of Label Enzymes

Example 1

A typical reaction solution for amplified detection of alkaline phosphatase consists of

2-methyl-2-amino-1-propanol buffer	0.1M, pH 9.4
$Mg(OAc)_2$	8.8×10^{-4} M
H_2O_2	0.3% (v/v)
luminol	1×10^{-3} M
p-phenylphenol phosphate	1×10^{-3} M
horseradish peroxidase-IgG	($1x10^{-10}$ - $1x10^{-18}$ mol)

Typically, 200 µL of the reagent was transferred into a polypropylene tube placed in a Turner TD 20e or other suitable luminometer. The background luminescence was measured for a period of 5 minutes after which time a 5 µL aliquot of an alkaline phosphatase solution was added. Figure 1 shows a plot of light intensity vs. time produced in this reaction at 25°C. This method allowed the measurement of as little as 6 x 10^{-18} mol of alkaline phosphatase in 205 µL with a signal/background ratio of 4.

Example 2

The solution of Example 1 was used with the addition of 0.1% non-fat dry milk (NFM) and the results were similar to Example 1 except that the background was significantly reduced.

Example 3

The solution of Example 1 was used with the addition of 5% of I-Block solution and the results were similar to Example 1 except that the background was significantly reduced.

Example 4

The solution of Example 1 was used with the substitution of p-iodophenyl phosphate for p-phenylphenol phosphate and the results were similar to Example 1.

Example 5

A typical reagent solution for amplified detection of beta-galactosidase consists of

phosphate buffer	0.1M, pH 7.6
$Mg(OAc)_2$	8.8×10^{-4} M
H_2O_2	0.3% (v/v)
luminol	1×10^{-3} M
p-phenylphenol galactoside	1×10^{-3}
horseradish peroxidase-IgG	10^{-10} - 10^{-18} mol)
non-fat dry milk (NFM)	0.1% (w/v)

Typically, 200 µL of the reagent was transferred into a polypropylene tube placed in a Turner TD 20e or other suitable luminometer. The background luminescence was measured for a period of 5 minutes after which time a 5 µL aliquot of a beta-galactosidase solution was added. Emission of chemiluminescence began immediately, reaching a maximum in less than 5 minutes, the intensity being proportional to the amount of beta-galactosidase. The light intensity decayed slowly over more than one hour.

Example 6

The solution of Example 5 was used with the substitution of p-iodophenyl galactoside for p-phenylphenol galactoside and the results were similar to Example 5.

Example 7

A typical reagent solution for amplified detection of esterase consists of

phosphate buffer,	0.1 M, pH 7.6
$Mg(OAc)_2$	8.8×10^{-4} M
H_2O_2	0.3 % (v/v)
luminol	1×10^{-3} M
p-phenylphenol acetate	1×10^{-3} M
horseradish peroxidase-IgG	10^{-10} - 10^{-18} mol
non-fat dry milk (NFM)	0.1% (w/v)

Typically, 200 µL of the reagent was transferred into a polypropylene tube placed in a Turner TD 20e or other suitable luminometer. The background luminescence was measured for a period of 5 minutes after which time a 5 µL aliquot of an esterase solution was added. Emission of chemiluminescence began immediately, reaching a maximum in less than 5 minutes, the intensity being proportional to the amount of esterase. The light intensity decayed slowly over more than one hour.

Chemiluminescent Detection of Proteins by Western Blot

Goat non-immune serum, human whole serum and rabbit anti-goat IgG-alkaline phosphatase conjugate were obtained from Cappel™ Products (Durham, NC). Human transferrin, fractionated goat anti-human transferrin serum, and nitrocellulose membrane were purchased from Sigma Chemical Co. (St. Louis, MO). Each serum and IgG sample was centrifuged at 10,000 g for two minutes and the supernatant was used in the immunological reaction. Immobilon™-P transfer membrane and nylon transfer membrane were obtained from Millipore Corp. (Bedford, MA) and MSI (Micron Separations, Inc., Westboro, MA), respectively. Kodak X-OMAT AR X-ray film (Rochester, NY) was used in the assay procedure.

SDS-PAGE was performed utilizing the buffer system previously described (U. K. Laemmli, Nature, 227, 680 (1970)). The stacking gel was 4.38% acrylamide:0.12% bisacrylamide. The separating gel was 6.81% acrylamide; 0.19% bisacrylamide. Following electrophoresis, the gel was equilibrated for 7-8 minutes with the transfer buffer which contained 20 mM Tris, 153 mM glycine and 20% (v/v) methanol. The gel, sandwiched between a sheet of Immobilon™-P transfer membrane and a sheet of chromatography paper 3MM (Whatman), was placed in the transfer unit (Bio-Rad Laboratories, Richmond, CA). The proteins in the gel were electroeluted for 60-80 minutes at 4°C at a 100 V constant voltage. The membrane was then placed in 50 mM Tris-HCl buffered saline at pH 7.4 (TBS) at 4°C overnight. After this period the membrane was washed with TBS for 15 minutes.

The membrane was treated with 0.05% Tween-20 in 50mM Tris-HCl buffered saline at pH 7.4 (T-TBS) containing 1% NFM for 1 hour at room temperature. This blocked membrane was incubated for 75 minutes at room temperature with primary antibody (1:500 dilution of goat anti-human transferrin IgG fraction) using T-TBS containing 1% NFM.

The membrane was incubated for 1 hour at room temperature with secondary antibody (1:5000 dilution of rabbit anti-goat IgG-alkaline phosphatase conjugate) in T-TBS containing 1% NFM. After the incubation, the membrane was rinsed and washed four times for 10 minutes each with T-TBS followed by a 10 minute wash in TBS. The membrane was rinsed three times with distilled water.

The washed membrane was soaked in the reagent solution for 5 minutes, drained, placed in a holder (transparency film) and the membrane exposed to X-ray film for a period ranging between 5 seconds and 30 minutes and then developed. Figure 2 shows a chemiluminescent Western slot blot wherein different concentrations of human transferrin bound to an antibody conjugated to alkaline phosphatase on an Immobilon™-P nylon membrane are contacted with the chemiluminescent reagent solution described in Example 1.

This chemiluminescent method allowed the measurement of 500 femtograms (5×10^{-13})/slot of human transferrin. Furthermore the method provides a non-fading permanent experimental record on X-ray film and makes it possible to safely and conveniently detect small amounts of protein with very short exposure times.

It is intended that the foregoing description be only illustrative of the present invention and that the invention be limited only by the hereinafter appended claims.

Claims

1. A method for generating light which comprises mixing:
   (a) a hydrolytic enzyme; and

(b) a protected enhancer compound, a peroxide compound, an amino substituted acylhydrazide which generates light upon reaction with the peroxide compound and the peroxidase enzyme, wherein the enhancer compound has the formula ArOX wherein X is a leaving group which is reactive with the hydrolytic enzyme and Ar is a non-interfering aromatic ring group which can contain C, O, S or N in the ring, wherein the hydrolytic enzyme reacts with the protected enhancer compound to remove X and thereby enhance the level of light produced by the amino substituted acylhydrazide as compared to the level of light without the enhancer compound.

2. The method of Claim 1 wherein the hydrolytic enzyme is attached to a biological molecule.

3. The method of Claim 1 wherein the hydrolytic enzyme is attached to an antibody.

4. The method of Claim 1 wherein the hydrolytic enzyme is attached to a molecule which is part of a binding pair in an assay.

5. The method of Claim 1 wherein the hydrolytic enzyme is attached to a nucleic acid.

6. The method of Claim 1 wherein the mixing and detecting is on a membrane.

7. The method of Claim 1 wherein the membrane is Immobilon™-P.

8. The method of Claim 1 wherein a suppressing agent is included to retard the generation of light before the addition of the hydrolytic enzyme.

9. The method of Claim 1 wherein OX is selected from the group consisting of alkyl carboxyl ester, carboxyl ester, inorganic oxyacid salt and oxygen pyranoside substituents.

10. The method of Claim 1 wherein the hydrolytic enzyme is selected from the group consisting of alkaline phosphatase, beta-galactosidase, and esterase.

11. A kit for detecting a hydrolytic enzyme in an assay which comprises a protected enhancer compound, a peroxide compound, a peroxidase and an amino substituted acylhydrazide which generates light upon reaction with the peroxide compound and the peroxidase enzyme, wherein the protected enhancer compound has the formula ArOx wherein X is a leaving group which is reactive with the hydrolytic enzyme and Ar is a non-interfering aromatic ring group which can contain C, O, S or N in the ring, wherein the hydrolytic enzyme reacts with the protected enhancer compound to remove X and thereby enhance the level of light produced by the amino substituted acylhydrazide as compared to the level of light without the enhancer compound.

12. The kit of Claim 11 which in addition includes a membrane on which the hydrolytic enzyme is detected.

13. The kit of Claim 11 which in addition includes the hydrolytic enzyme coupled to one member of a binding pair in the assay.

14. The kit of Claim 11 which in addition includes an antibody coupled to the hydrolytic enzyme.

15. The kit of Claim 11 which in addition includes an antigen coupled to the hydrolytic enzyme.

16. The kit of Claim 11 which in addition includes a nucleic acid coupled to the hydrolytic enzyme.

17. The kit of Claim 11 wherein a suppressing agent which retards the generation of light without the hydrolytic enzyme is also included.

18. The kit of Claim 17 wherein the suppressing agent is selected from the group consisting of proteins and surfactants.

19. The kit of Claim 11 wherein OX is selected from the group consisting of alkyl carboxyl ester, carboxyl ester, inorganic oxyacid salt and oxygen pyranoside substituents.

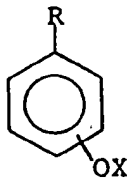
20. A composition for reaction with a hydrolytic enzyme which comprises:
(a) an amino substituted acylhydrazide; and
(b) a protected enhancer compound having the formula ArOX wherein X is a leaving group which is reactive with the hydrolytic enzyme and Ar is a non-interfering aromatic group which can contain C, O, S or N in the ring, wherein the hydrolytic enzyme reacts with the protected enhancer compound to remove X and thereby enhances the level of light produced by the amino substituted acylhydrazide in the presence of a peroxide compound and a peroxidase as compared to the level of light without the enhancer compound.

21. The composition of Claim 20 in admixture with the peroxidase and the peroxide compound with a suppressing agent.

22. The composition of Claim 21 wherein the suppressing agent is selected from the group consisting of proteins and surfactants.

23. The composition of Claim 20 wherein OX is selected from the group consisting of alkyl carboxyl ester, carboxyl ester, inorganic oxyacid salt and oxygen pyranoside substituents.

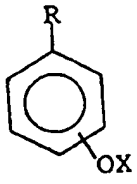
24. A method for generating light which comprises mixing:
(a) a hydrolytic enzyme; and
(b) a protected enhancer compound, a peroxide compound and an amino substituted acylhydrazide which generates light upon reaction with the peroxide compound and the peroxidase enzyme, wherein the enhancer compound has the formula



wherein X is a leaving group which is reactive with the hydrolytic enzyme and R is a non-interfering group, wherein the hydrolytic enzyme reacts with the protected enhancer compound to remove X and thereby enhance the level of light produced by the amino substituted acylhydrazide as compared to the level of light without the enhancer compound.

25. The method of Claim 24 which in addition includes a suppressing agent to retard the generation of light in the absence of the hydrolytic enzyme.

26. A method for detecting the presence of a hydrolytic enzyme which comprises:
(a) mixing the hydrolytic enzyme, a protected enhancer compound, a peroxidase, a peroxide compound and an amino substituted acylhydrazide which generates light upon reaction with peroxide compound and the peroxidase, wherein the protected enhancer compound has the formula



wherein X is a leaving group which is removed by the hydrolytic enzyme and R is a non-interfering organic group, wherein the hydrolytic enzyme reacts with the protected enhancer compound to remove X and thereby enhance the level of light produced by the amino substituted acylhydrazide as compared to the level of light without the enhancer compound; and

(b) detecting the hydrolytic enzyme, wherein the quantity of enzyme is a function of the light produced by the amino substituted acylhydrazide.

27. The method of Claim 26 which in addition includes a suppressing agent to retard the generation of light in the absence of the hydrolytic enzyme.

28. The method of Claim 26 wherein the hydrolytic enzyme is attached to a biological molecule.

29. The method of Claim 26 wherein the hydrolytic enzyme is attached to an antibody.

30. The method of Claim 26 wherein the hydrolytic enzyme is attached to a molecule which is part of a binding pair in an assay.

31. The method of Claim 26 wherein the hydrolytic enzyme is attached to a nucleic acid.

32. The method of Claim 26 wherein the mixing and detecting is on a membrane.

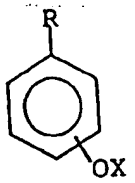
33. The method of Claim 32 wherein the membrane is Immobilon™-P.

34. The method of Claim 24 wherein OX is selected from the group consisting of alkyl carboxyl ester, carboxyl ester, inorganic oxyacid salt and oxygen pyranoside substituents.

35. The method of Claim 24 wherein the hydrolytic enzyme is selected from the group consisting of alkaline phosphatase, beta-galactosidase and esterase.

36. The method of Claim 26 wherein the hydrolytic enzyme is selected from the group consisting of alkaline phosphatase, beta-galactosidase and esterase.

37. A kit for detecting a hydrolytic enzyme in an assay which comprises a protected enhancer compound, a peroxide compound, a peroxidase and an amino substituted acylhydrazide which generates light upon reaction with the peroxide compound and the peroxidase enzyme, wherein the protected enhancer compound has the formula



wherein X is a leaving group which is reactive with the hydrolytic enzyme and R is a non-interfering group, wherein the hydrolytic enzyme reacts with the protected enhancer compound to remove X and thereby enhance the level of light Produced by the amino substituted acylhydrazide as compared to the level of light without the enhancer compound.

38. The kit of Claim 37 which in addition includes a membrane on which the hydrolytic enzyme is detected.

39. The kit of Claim 37 which in addition includes the hydrolytic enzyme coupled to one member of a binding pair in the assay.

40. The kit of Claim 37 which in addition includes an antibody coupled to the hydrolytic enzyme.

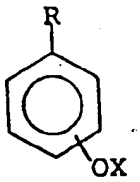
41. The kit of Claim 37 which in addition includes an antigen coupled to the hydrolytic enzyme.

42. The kit of Claim 37 wherein a suppressing agent which retards the generation of light without the hydrolytic enzyme is also included.

43. The kit of Claim 42 wherein the suppressing agent is a protein.

44. The kit of Claim 37 wherein OX is selected from the group consisting of alkyl carboxyl ester, carboxyl ester, inorganic oxyacid salt and oxygen pyranoside substituents.

45. A composition for reaction with a hydrolytic enzyme which comprises:
(a) an amino substituted acylhydrazide; and
(b) a protected enhancer compound having the formula



wherein X is a leaving group which is reactive with the hydrolytic enzyme and R is a non-interfering group, wherein the hydrolytic enzyme reacts with the protected enhancer compound to remove X and thereby enhances the level of light produced by the amino substituted acylhydrazide in the presence of a peroxide compound and a peroxidase as compared to the level of light without the enhancer compound.

46. The composition of Claim 45 in admixture with the peroxidase and the peroxide compound with a suppressing agent.

47. The composition of Claim 45 wherein the suppressing agent is a protein.

48. The composition of Claim 45 wherein OX is selected from the group consisting of alkyl carboxyl ester, carboxyl ester, inorganic oxyacid salt and oxygen pyranoside substituents.

49. The method of Claim 24 wherein R is selected from the group consisting of halogen, alkyl containing 1 to 30 carbon atoms, aryl containing 1 to 30 carbon atoms, aralkyl containing 1 to 30 carbon atoms and wherein alkyl, aryl or aralkyl can be substituted with a halogen wherein aryl can include O, N or S substituted for a carbon and wherein OX is para to R.

50. The method of Claim 26 wherein R is selected from the group consisting of a halogen, alkyl containing 1 to 30 carbon atoms, aryl containing 1 to 30 carbon atoms, aralkyl containing 1 to 30 carbon atoms and wherein alkyl, aryl or aralkyl can be substituted with a halogen and wherein OX is para to R.

51. The kit of Claim 37 wherein R is selected from the group consisting of a halogen, alkyl containing 1 to 30 carbon atoms, aryl containing 1 to 30 carbon atoms, aralkyl containing 1 to 30 carbon atoms and wherein alkyl, aryl or aralkyl can be substituted with a halogen and wherein OX is para to R.

52. The composition of Claim 45 wherein R is selected from the group consisting of a halogen, alkyl containing 1 to 30 carbon atoms, aryl containing 1 to 30 carbon atoms, aralkyl containing 1 to 30 carbon atoms and wherein alkyl, aryl or aralkyl can be substituted with a halogen and wherein OX is para to R.

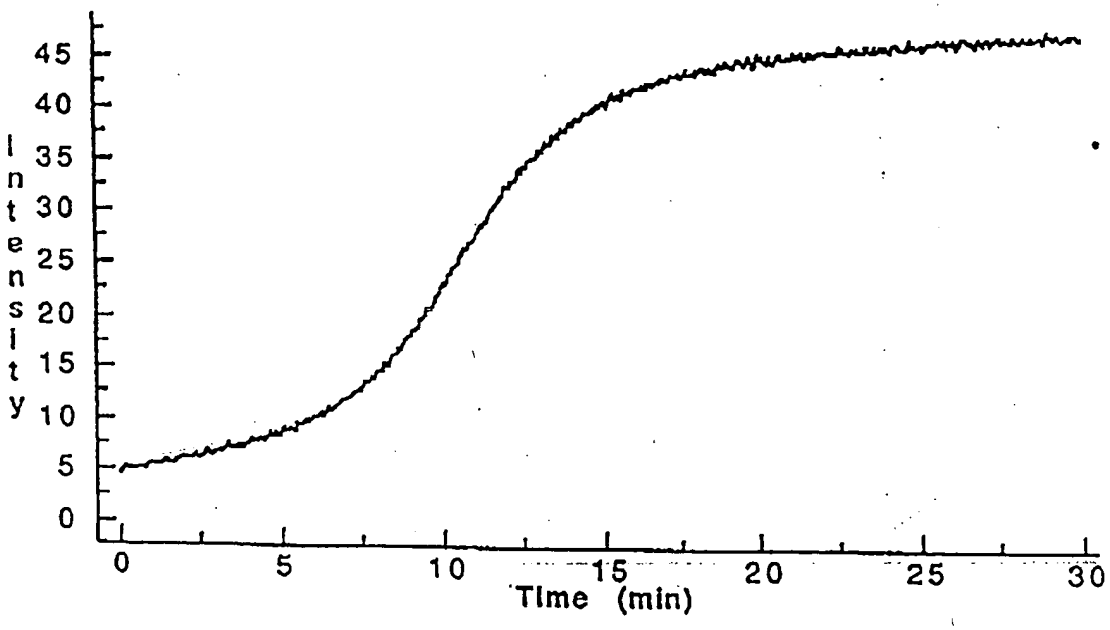


FIGURE 1

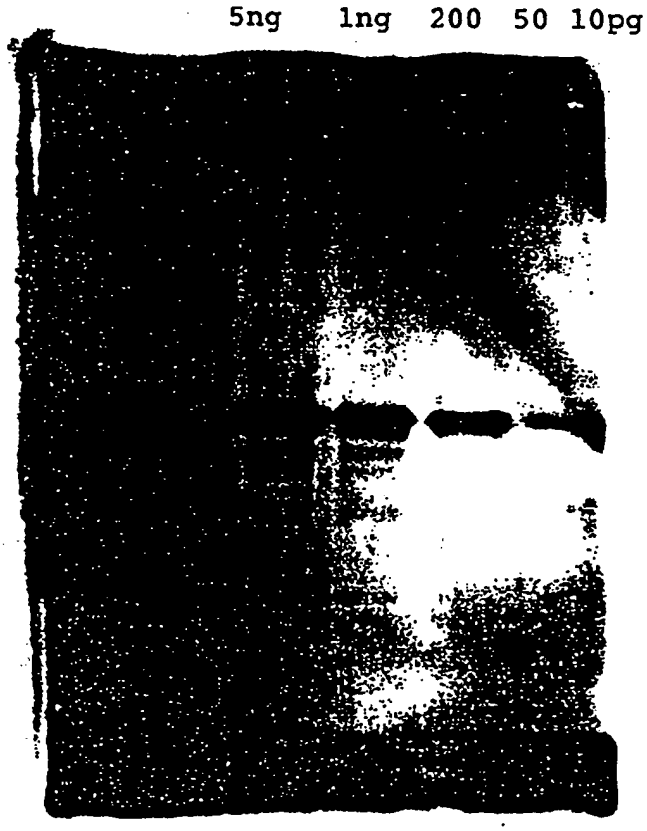


FIGURE 2

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 92 10 6544

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)

X	WO-A-9 105 872 (NATIONAL RESEARCH DEVELOPMENT CORPORATION)
	* the whole document *
		1,2-5, 9-11, 14-16, 19,20, 23,24, 26,28-31 34-37, 39-41, 44-52	G01N33/58
			//G01N21/27
			//C12Q1/28
			//C12Q1/34
			//C12Q1/68
			//C12Q1/42
			//C12Q1/44
			//G01N33/53
			//G01N33/535
			//G01N33/68

D,A	ANALYTICAL BIOCHEMISTRY vol. 151, 1985, NEW YORK US pages 205 - 209; J.A.MATTHEWS ET AL.: 'Enhanced chemiluminescent method for the detection of DNA dot-hybridization assays' * page 206 - page 207 *	1,6,7, 12,32, 33,38	
A	EP-A-0 087 959 (SECRETARY OF STATE FOR SOCIAL SERVICES) * page 1 - page 5 *	1,9,19, 34,43	
A	EP-A-0 116 454 (NATIONAL RESEARCH DEVELOPMENT CORPORATION) * page 3, line 28 - page 5, line 10 * * page 7, line 9 - line 29 *	1,9,19, 34,43	TECHNICAL FIELDS SEARCHED (Int. Cl.5) G01N C12Q
A	EP-A-0 296 752 (NATIONAL RESEARCH DEVELOPMENT CORPORATION) * the whole document *	1,9,19, 34,43	
A	EP-A-0 361 470 (FUJIREBO INC.) * the whole document *	1,9,19, 34,43	
A	EP-A-0 384 271 (TAKEDA CHEMICAL INDUSTRIES, LTD.) * the whole document *	1,9,19, 34,43	

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	25 SEPTEMBER 1992	DE KOK A.J.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P0601)

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 92 10 6544

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	US-A-4 931 385 (E.BLOCK ET AL.) * column 14, line 10 - line 27 *	8,17,25, 27,42	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	25 SEPTEMBER 1992	DE KOK A.J.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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